

**COMPARATIVE PHENOMICS AND GENOMICS
OF Carbapenem-resistant *Escherichia coli* FROM
HUMAN AND BROILER CHICKEN**

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OF Carbapenem-resistant *Escherichia coli* FROM
HUMAN AND BROILER CHICKEN**

by

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TABLE OF CONTENTS

ACKNOWLEDGMENT	ii
TABLE OF CONTENTS.....	iii
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xxii
LIST OF APPENDICES	xxxii
ABSTRAK	xxxii
ABSTRACT.....	xxxiv
CHAPTER 1 INTRODUCTION.....	1
1.1 Background of the study	1
1.2 Justification of the study	6
1.3 Research questions	6
1.4 Objectives of the study.....	7
1.4.1 General objectives.....	7
1.4.2 Specific objectives	7
CHAPTER 2 LITERATURE REVIEW.....	8
2.1 General characteristics of <i>Escherichia coli</i>	8
2.2 Pathogenic <i>Escherichia coli</i>	9
2.3 Antibiotics discovery and the ensuing medical revolution	10
2.4 Mechanisms of action of antibiotics	12
2.4.1 Interfering with cell wall synthesis	13
2.4.2 Inhibiting of protein synthesis.....	14

2.4.3	Inhibition of nucleic acid synthesis	15
2.4.4	Inhibition of metabolic pathways/bacterial enzymes	16
2.4.5	Interruption of bacterial membrane.....	17
2.5	Antimicrobial Resistance	18
2.5.1	Mechanisms of antimicrobial resistance	20
2.5.1(a)	Genetic basis of antimicrobial resistance	20
2.5.1(b)	Mutational resistance.....	21
2.5.1(c)	Horizontal gene transfer	21
2.5.1(d)	Mechanistic basis of antimicrobial resistance	23
2.5.1(e)	Drug uptake limitation	24
2.5.1(f)	Drug target modification	27
2.5.1(g)	Drug inactivation.....	28
2.5.1(h)	Drug efflux.....	29
2.6	Carbapenem antibiotics	31
2.6.1	Historical overview: the discovery and use of carbapenems	31
2.6.2	Structural features and functions.....	32
2.6.3	Mode of actions of carbapenems.....	34
2.6.4	Usage and side-effects of carbapenems	35
2.7	Carbapenem resistance and carbapenemases	37
2.7.1	Mechanisms of carbapenem resistance	38
2.7.1(a)	Expression of efflux pumps	38
2.7.1(b)	Decreased outer membrane permeability via porin mutations	39
2.7.1(c)	Enzymatic degradation through carbapenemase production	40
2.7.2	Classification of carbapenemases	41

2.7.2(a)	Class A carbapenemases	41
2.7.2(b)	Class B carbapenemases	42
2.7.2(c)	Class D carbapenemases	45
2.8	Carbapenem-resistant Enterobacterales	48
2.8.1	Carbapenem-resistant <i>Escherichia coli</i>	50
2.8.2	Global epidemiology of carbapenem-resistant Enterobacterales.....	51
2.8.3	Carbapenem-resistant Enterobacterales in Southeast Asia.....	55
2.8.4	Carbapenem-resistant Enterobacterales in Malaysia.....	59
2.8.5	Spread and transmission of carbapenem-resistant Enterobacterales and the associated risk factors	60
2.8.6	Treatment of infections caused by carbapenem-resistant Enterobacterales	62
2.9	Carbapenem-resistant Enterobacterales in animals	64
2.10	Livestock as a potential source of carbapenem-resistant Enterobacterales infection or colonization in humans	68
2.11	Antibiotic usage in animals and its role in the emergence and spread of carbapenem-resistant Enterobacterales	69
2.12	Laboratory detection of carbapenem-resistant Enterobacterales	74
2.12.1	Phenotypic methods	75
2.12.1(a)	Antimicrobial susceptibility testing	75
2.12.1(b)	Double disk synergy tests (DDST)	77
2.12.1(c)	Chromogenic-based media for CRE screening.....	79
2.12.1(d)	Carbapenem inactivation method (CIM)	81
2.12.1(e)	Modified Hodge test (MHT)	82

2.12.1(f) Carba NP	83
2.12.1(g) Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)	84
2.12.2 Molecular methods.....	85
2.12.2(a) Conventional PCR methods.....	87
2.12.2(b) Real-time PCR methods.....	89
2.12.2(c) Hybridization-based techniques (Microarrays)	92
2.12.2(d) Whole genome sequencing	93
2.13 Comparative genomics of carbapenem-resistant Enterobacterales	93

**CHAPTER 3 ISOLATION, IDENTIFICATION, AND ANTIMICROBIAL
RESISTANCE PROFILES OF CARBAPENEM-RESISTANT *E. coli***

ISOLATED FROM HUMAN AND BROILER CHICKEN	98
3.1 Introduction.....	98
3.2 Objectives of the study.....	102
3.3 Materials and methods	103
3.3.1 Study design.....	103
3.3.2 Ethics statement.....	103
3.3.3 Human clinical <i>E. coli</i> isolates	103
3.3.4 Study area, sampling method, and sample size determination.....	104
3.3.5 Isolation and identification of <i>E. coli</i>	105
3.3.6 PCR confirmation of <i>E. coli</i>	106
3.3.7 Antibiotic susceptibility test	107
3.3.8 Determination of multiple antimicrobial resistance (MAR) index.....	107
3.3.9 Screening of CREC using chromogenic media, Brilliance™ CRE	

agar.....	108
3.3.10 Modified Hodge test (MHT)	108
3.3.11 Determination of minimum inhibitory concentration (MIC) using E-Test	109
3.3.12 Modified carbapenem inactivation method (mCIM) for carbapenemase detection.....	109
3.3.13 Statistical analysis	110
3.4 Results.....	111
3.4.1 Prevalence of carbapenemase-positive <i>E. coli</i> in poultry from Kelantan, Terengganu, and Pahang.....	112
3.4.2 Antimicrobial resistance profiles of CREC isolates from human clinical cases and poultry	111
3.4.3 Multiple antimicrobial resistance (MAR) index	113
3.4.4 Screening of CREC using chromogenic media (Brilliance CRE Agar)	114
3.4.5 Modified Hodge test (MHT)	115
3.4.6 Determination of minimum inhibitory concentration by E-test.....	115
3.4.7 Modified carbapenem inactivation method (mCIM)	116
3.2 Discussions.....	119
3.3 Conclusion	128
CHAPTER 4 MOLECULAR CHARACTERIZATION OF CARBAPENEM- RESISTANT <i>E. coli</i>	130
4.1 Introduction.....	130
4.2 Objectives of the study.....	134

4.3	Materials and methods	135
4.3.1	Bacterial isolates	135
4.3.2	Multiplex PCR detection of carbapenem resistance encoding genes.....	135
4.3.3	Multiplex PCR for detection of virulence genes in CREC isolates	137
4.3.4	Phylotyping of CREC isolates	138
4.3.5	Statistical analysis	140
4.4	Results	141
4.4.1	Detection of carbapenem resistance encoding genes	141
4.4.2	Detection of virulence genes	143
4.4.3	Phylogenetic analysis	145
4.5	Discussions.....	152
4.6	Conclusion	159
 CHAPTER 5 MOLECULAR EPIDEMIOLOGY OF CARBAPENEM- RESISTANT <i>E. coli</i> ISOLATED FROM HUMANS AND BROILER CHICKEN.....		
		161
5.1	Introduction.....	161
5.2	Objectives of the study	163
5.3	Materials and Methods	164
5.3.1	Bacterial isolates	164
5.3.2	PCR for detection of housekeeping genes of <i>E. coli</i>	164
5.3.3	Sequencing, sequence analysis, and typing of CREC isolates.....	165
5.3.4	Data analysis	166
5.4	Results	167
5.5	Discussions.....	179

5.6	Conclusion	185
CHAPTER 6 COMPARATIVE GENOMICS OF Carbapenem-resistant <i>E. coli</i>		
ISOLATED FROM HUMANS AND BROILER CHICKENS..... 186		
6.1	Introduction	186
6.2	Objectives of the study	190
6.3	Materials and Methods	191
6.3.1	Strain selection	191
6.3.2	Genomic DNA extraction	192
6.3.3	Genome sequencing	192
6.3.4	Library preparation.....	192
6.3.4(a)	DNA qualification and library construction	193
6.3.4(b)	Library quality control.....	194
6.3.4(c)	High-throughput DNA sequencing.....	194
6.3.5	Bioinformatics analyses	195
6.3.5(a)	Quality control and de novo assembly.....	195
6.3.5(b)	Genome sequence analysis and annotation	196
6.3.5(c)	Miscellaneous comparative genomic analysis	197
6.4	Results	199
6.4.1	Genome assembly and annotation statistics and quality	199
6.4.2	Open reading frames (ORFs), orthologous groups, orthologous sequences alignment, and phylogenetic tree reconstruction	200
6.4.3	Antibiotics resistance genotypes	203
6.4.4	Virulence gene profiles	204
6.4.5	Plasmids and pMLST profiles.....	205

6.4.6	Serotype, Fimtype, CHtype, and cgMLST profiles	206
6.4.7	SNP-based phylogenetic analyses.....	213
6.4.8	Phage-associated gene clusters	224
6.4.9	Miscellaneous comparative genomics of the CREC isolates	226
6.5	Discussions.....	246
6.6	Conclusion	255
CHAPTER 7 GENERAL DISCUSSIONS		256
CHAPTER 8 CONCLUSION AND FUTURE DIRECTION		264
8.1	Conclusion	264
8.2	Future direction.....	270
REFERNCES		271
APPENDICES		
PUBLICATIONS		

LIST OF TABLES

	Page
Table 2.1	<i>Escherichia coli</i> pathotypes and their main characteristics 11
Table 2.2	Classification of β -Lactamases (Opal and Pop-Vicas, 2020).....47
Table 2.3	Bush-Jacoby-Medeiros Functional Classification Scheme for β -Lactamases.....48
Table 2.4	Breakpoints for carbapenems against Enterobacterales family according to EUCAST (2022) and CLSI (2021) guidelines 76
Table 3.1	Prevalence and distribution of CPEC in poultry cloacal samples collected from Kelantan, Terengganu, and Pahang 112
Table 3.2	Phenotypic resistance characteristics of CREC isolates from human clinical cases and poultry cloacal swabs 117
Table 4.1	Primers for amplification of <i>E. coli</i> -specific and carbapenem Resistance encoding genes (Poirel <i>et al.</i> , 2011)..... 136
Table 4.2	Primer sequences used for multiplex PCR detection of virulence genes of CREC isolates from human and poultry 139
Table 4.3	Primer sequences used for the detection of phylogroups of <i>E. coli</i> isolates using quadruplex PCR and allele-specific PCR..... 140
Table 4.4	Phylogenetic group, resistance, and virulence genes of CREC isolates from human and poultry 149
Table 5.1	Primer sequence of singleplex PCR used for the detection of <i>E. coli</i> housekeeping genes for MLST in CREC isolates..... 165
Table 6.1	Phenotypic and genotypic characteristics of CREC isolates selected for WGS 191

Table 6.2	Genome statistics of whole genome draft sequences of CREC isolates from humans and poultry	200
Table 6.3	Annotation statistics of whole genome draft sequences of CREC isolates from humans and poultry	200
Table 6.4	Genome quality of whole genome draft sequences of CREC isolates from humans and poultry	200
Table 6.5	Genomic characteristics of CREC isolates from humans and poultry ...	207
Table 6.6	Global epidemiology of close isolates related to CREC isolates from poultry and human (*) based on cgMLST	209
Table 6.7	Diversity in multiple genome alignment.....	217
Table 6.9	Prophage regions of CREC genomes assigned as intact, questionable, or incomplete based on the similarity score generated by PHASTER (http://phaster.ca/).....	224
Table 6.10	Mobile genetic elements and plasmids associated with resistance and virulence genes of CREC isolates from human and poultry	237

LIST OF FIGURES

	Page
Figure 2.1	Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents. Adapted from (Mulvey and Simor, 2009) 18
Figure 2.2	Antibiotic targets and mechanisms of resistance. Adapted from (Wright, 2010)..... 24
Figure 2.3	The R configuration of the hydroxyethyl groups in the beta-lactam. The R configuration of the hydroxyethyl of the beta-lactam (Papp-Wallace <i>et al.</i> , 2011)..... 33
Figure 2.4	Chemical structure of Penicillin, cephalosporin, and carbapenem backbones. Carbapenem has a five-membered ring, as does penicillin, but it has a carbon at C-1 instead of sulfur. Adapted from (Papp-Wallace <i>et al.</i> , 2011) 33
Figure 2.5	Chemical structures of carbapenem antibiotics..... 34
Figure 2.6	Global distribution of carbapenemases in Enterobacterales by country and region. Adapted from (Logan and Weinstein, 2017)..... 55
Figure 2.7	Prevalence of carbapenem-resistant <i>E. coli</i> and <i>Klebsiella</i> spp. in Southeast Asia. Adapted from (Malchione <i>et al.</i> , 2019)..... 57
Figure 2.8	Estimated median national carbapenem resistance proportions for (A) <i>E. coli</i> and (B) <i>Klebsiella</i> spp. based on including samples from 2010–2017. Adapted from (Malchione <i>et al.</i> , 2019)..... 58
Figure 2.9	Carbapenem and polymyxin(s) resistance genotypes reported from Southeast Asia Adapted from (Malchione <i>et al.</i> , 2019)..... 58

Figure 2.10	Global distribution of carbapenem-resistant and carbapenemase-producing Enterobacterales in (A) livestock and aquatic animals, and (B) companion animals and wildlife. Adapted from (Kock <i>et al.</i> , 2018).....	67
Figure 2.11	Global antimicrobial consumption in livestock in milligrams per 10 km ² pixels (Top) and average SD of estimates of milligrams per PCU (Bottom). Adapted from (Van Boeckel <i>et al.</i> , 2015).	71
Figure 2.12	Antimicrobial consumption in chickens (A) and pigs (B) in 2010. Purple indicates new areas where antimicrobial consumption will exceed 30 kg per 10 km ² by 2030. Adapted from (Van Boeckel <i>et al.</i> , 2015).	71
Figure 3.1	Location of the study area. The samples were collected from 10 districts in Kelantan, Terengganu, and Pahang, off the East Coast of Peninsular Malaysia. The map was created using ArcGIS v. 7 (Esri Inc., Redlands, CA, USA).....	105
Figure 3.2	Antibiotic resistance profiles of carbapenemases positive <i>E. coli</i> isolates from human and broiler chicken	113
Figure 3.3	Multiple antimicrobial resistance (MAR) and MAR index of CREC isolates from human and broiler chicken	114
Figure 3.4	MIC values CREC isolates from humans and poultry show values ranging from 0.25µg/ml to ≥256µg/ml (marked by 33*) for doripenem, ertapenem, imipenem, and meropenem	116
Figure 4.1	Multiplex PCR amplification of carbapenemase genes of CREC isolates.....	142

Figure 4.2	Distribution of carbapenemases genes in CREC isolates from human and broiler chicken	142
Figure 4.3	Amplification products from multiplex PCR detection of virulence genes detection in CREC isolates showing positive results for <i>astA</i> , <i>iss</i> , <i>irp2</i> , and <i>iucD</i> genes.....	144
Figure 4.4	Prevalence of virulence genes in CREC and their distribution according to the source (human and broiler chicken)	144
Figure 4.5	Prevalence of virulence genes of CREC isolates from human and broiler chicken and their distribution based on the associated phylogenetic groups	145
Figure 4.6	Quadruplex PCR profiles of the new Clermont phylotyping method. group A (P3, P4, P5, P6, P7, P8, + - - -) group B1 (P1, P11, P13, P18, + - - +), group C (P2, P9, P16, + - + -), group E (P10, + + - -); unknown (P17, - + - -); group D (P10, + + - -); Lanes EF1 and EF2 (<i>Escherichia fergusonii</i> - - - -).	146
Figure 4.7	Distribution of phylogenetic groups in CREC isolates from human and broiler chicken.....	147
Figure 4.8	Distribution of carbapenemases genes and the associated phylogenetic groups of CREC isolates from human and broiler chicken	147
Figure 4.9.	Phylogenetic groups of CREC, their association with virulence genes and distribution according to the source (human and broiler chicken)	148

Figure 5.1	The phylogenetic relationship between sequence types and clonal complexes of CREC isolates from humans and broiler chicken	168
Figure 5.2	Phylogenetic tree showing the relationship among the different CREC isolates and their evolution (constructed using Interactive Tree of Life (iTOL) v5)	169
Figure 5.3	Distribution of STs and clonal relationships of CREC isolates according to the sources	170
Figure 5.4	Distribution of sequence type (STs) belonging to 8 clonal complexes (CCs) and 11 singletons of CREC isolates from humans and poultry	171
Figure 5.5	Field breakdown analysis of <i>E. coli</i> dataset (https://pubmlst.org) including 4774 STs (A) and 51 CCs (B) based on Achtman MLST scheme analysis showing ST131 Cplx, ST23 Cplx, ST155 Cplx, and ST69 Cplx as the top ten most prevalent <i>E. coli</i> clonal complexes globally.....	172
Figure 5.6	Phylogenetic relationship of STs and the carbapenemases genes of CREC isolates from humans and poultry	173
Figure 5.7	Phylogenetic relationship of STs and the associated phylogroups of CREC isolate.....	174
Figure 5.8	Phylogenetic relationship of clonal complexes (CCs) and the associated phylogroups of CREC isolates.....	175
Figure 5.9	Global distribution of the STs of the CREC isolates based on pubmlst database (https://pubmlst.org) showing that the ST are distributed mainly in Ireland, China, and Brazil.	176

Figure 5.10	Distribution of the CREC STs isolated from humans and poultry, the evolutionary relationship among the STs and the four major sources of the <i>E. coli</i> STs based on pubmlst database (https://pubmlst.org)	177
Figure 5.11	MLST-sequence-based UPGMA-tree showing sequence types and clonal complexes of CREC isolates and their corresponding phenotypic and genotypic carbapenem resistance features. The numbers shown on the branches of the tree indicate the linkage distances.....	178
Figure 6.1	Workflow of library preparation	193
Figure 6.2	Experimental procedures of library preparation	194
Figure 6.3	Genomic data showing a) GC content per genome (51.4% - 51.8%), b) ORF content per genome, c) Orthologous group size, and d) phylogenetic tree of the CREC isolates with <i>E. fergusonii</i> ATCC 35466 as an out group.....	202
Figure 6.4	Comparison of <i>bla</i> _{NDM} variants (NDM-1 and NDM-4) on the subclass B1 beta-lactamase regions of CREC isolates from human (HEC15 and HEC25) and broiler chickens (KM7.P1 and KPM36.P15)	204
Figure 6.5	Global distribution of cgMLST-ST154483 CREC-related <i>E. coli</i> clones.....	210
Figure 6.6	Hosts/sources of cgMLST-ST154483 CREC-related <i>E. coli</i> clones and their phylogenetic relationships	211
Figure 6.7	Diseases associated with cgMLST-ST154483 CREC-related <i>E. coli</i>	

	and their phylogenetic relationships.....	212
Figure 6.8	Heatmap showing SNP-based similarity matrix among CREC isolates from broiler chickens and human and reference genomes. Intense maroon color (dark red) indicates more SNP count differences between the genomes (>39, 000). The intensity of color from white to dark red increases with the increasing divergence between the genomes compared.....	214
Figure 6.9	Heatmap showing SNP-based similarity matrix of CREC isolates from human and broiler chickens and reference genomes of CRE isolates from human and chicken. Genome identity between/among the isolates are shown by intense blue color, while closer similarity is represented by lighter blue color. Divergence among the genomes are represented by white and red colors. The heatmap was drawn by using Morpheus (https://software.broadinstitute.org/morpheus)	215
Figure 6.10	Phylogeny of CREC genomes <i>E. coli</i> str. K-2.substr.MG1655 was used as the reference genome, and other carbapenem-resistant <i>E. coli</i> from human and animals were included for comparison.....	216
Figure 6.11	Fractions of supporting vs clashing SNPs in relation to branch fractions.....	218
Figure 6.12	Trace of SNPs per 1 kb block along the core alignment.....	218
Figure 6.13	Cumulative distribution function (CDF) and histogram of pairwise distance distributions.....	218
Figure 6.14	Entropy profiles of the N-SNP distribution for each strain.....	219

Figure 6.15	Reverse cumulative distributions of the frequencies of all observed N-SNPs. The size (N) and the corresponding color are indicated in the legend	220
Figure 6.16	Probability distribution of the number of consecutive SNP columns that are consistent with a common phylogeny for the core genome alignment.....	220
Figure 6.17	Probability distribution of the number of consecutive alignment columns consistent with a common phylogeny	220
Figure 6.18.	Ratio C/S of the minimal number of phylogeny changes C to Substitutions S for random subsets of strains using the alignment from which 5% of potentially homoplastic positions have been removed. For strain numbers ranging from n=4, random subsets of n strains were collected, and the ratios C/S of phylogeny changes to SNPs in the alignment were calculated. The figure shows means and standard deviations for C/S across subsets.....	220
Figure 6.19	Pairwise SNP analysis of closely related CREC strains from human (HEC13 vs HEC17)	221
Figure 6.20	Pairwise SNP analysis of closely related CREC strains from human (HEC16) and poultry (PK7.P19)	222
Figure 6.21	Pairwise SNP analysis of closely related CREC strains from human (HEC15) and poultry (KPM36.P15)	223
Figure 6.22	Prophage regions of a CREC isolate, HEC15 harboring multiple antimicrobial resistance conferring genes.....	225
Figure 6.23	A representative complete (intact) prophages of CREC isolate,	

	HEC8 showing the detail structural features of the respective prophage regions in the genome	225
Figure 6.24	Pairwise alignment of selected CREC isolate from broiler chicken (A) and humans. Regions of genome similarity and homology were represented by colored blocks (LCBs), while the red lines represent contig/chromosome boundaries.....	227
Figure 6.25	Multiple sequence alignment of CREC genomes from human and broiler chicken. Sequences were aligned against the reference strain, <i>E. coli</i> str.K2substr.MG1655. Regions of genome similarity and homology were represented by colored blocks (LCBs), while the red lines represent contig/chromosome boundaries. The white regions or gaps between the LCBs correspond to sequences that are not aligned to the reference genome sequence that may contain sequence elements specific to a particular genome	228
Figure 6.26	Blast ring image showing genome similarities among the draft genomes of the CREC isolates. The legend on the side illustrates the color representation for each strain. Strains belonging to the same group have the same color. Different color tones indicate percentage of a genome region's identity to the reference genome	229
Figure 6.27	Fast ANI analyses of closely related CREC strains. Each red line segment denotes a reciprocal mapping between the query and reference genome, indicating their evolutionary conserved regions.....	230
Figure 6.28	Representative origin of transfers (OriT) in CREC isolates genome (HEC16) on MGEs encoding several predicted	

	antimicrobial resistance and virulence genes	232
Figure 6.29a	Comparative genomic island of CREC isolates showing island clusters carrying AMR genes	235
Figure 6.29b.	Genomic Island of CREC showing similar GI cluster (red) with AMR genes (pink) in all the CREC isolates	236
Figure 6.30	Comparison of mobile genetic elements (MGEs) and putative horizontal gene transfer (HGT) events of HEC15 (human) and KPM36.P15 (broiler chickens) CREC isolates. More MGEs are evident in HEC15 (A), whereas relatively more HGT events are observed in KPM36.P15 (B)	241
Figure 6.31	Proteome comparisons of CREC isolates. <i>E. coli</i> O25b:H4-ST131 strain 2822 was used as the reference genome. Overall, all the isolates share more than 80% protein sequence identity as compared to the majority of protein sequences in the reference genome.....	243
Figure 6.32	Comparative systems analyses showing PATRIC cross-genus specific (PLfams) of proteins in the CREC genomes	244
Figure 6.33	Comparative systems analyses showing PATRIC cross-genus specific(PLfams) of proteins in the CREC genomes	245

LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Definition
%	percentage
<	less than
>	more than
°C	degree Celsius
β -	Beta
g	gram
h	hour
ml	milliliter
pmol	picomole
pH	potential of hydrogen
s	seconds
μ g	microgram
μ l	microliter
μ g/ml	microgram/ milliliter
Zn ²⁺	Zinc ion
μ m	micromolar
ABC	ATP-binding cassette
AcrA	Acriflavine resistance A
AcrB	Acriflavine resistance B
<i>adk</i>	Adenylate kinase

<i>aer</i>	Aerobactin
<i>afa</i>	Afimbrial adhesins
AIM-1	Adelaide Imipenemase-1
AmpC	Ampicillin and carbenicillin
AMR	Antimicrobial resistance
ANI	Average Nucleotide Identity
ARGs	Antimicrobial resistance genes
<i>arpA</i>	Ankyrin-like regulatory protein
<i>ast</i>	Arginine succinyltransferase
ATCC	American Type Culture Collection
BacWGSTdb	Bacterial whole-genome sequence typing database
BHIA	Brain Heart Infusion agar
BIC-1	Bice ^{tre} carbapenemase
<i>bla</i> genes	Beta-lactamases genes
BLAST	Basic Local Alignment Search Tool
BRIG	BLAST Ring Image Generator
BSIs -	bloodstream infections
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CA	Clavulanate
CC	Clonal complex
CDC	Center for Disease Control
CDS	Coding sequences
cgMLST	Core genome multilocus sequence typing

<i>chuA</i> ,	<i>Escherichia coli</i> haem-utilization gene
CIM	Carbapenem inactivation method
CLSI	Clinical and Laboratory Standards Institute
<i>cnf1</i>	Cytotoxic necrotizing factor 1
CP-CRE	Carbapenemase-producing CRE
CPEC	Carbapenemase-producing <i>E. coli</i>
CPE	Carbapenemase-producing Enterobacterales
CRAB	Carbapenem resistant <i>A. baumannii</i>
CRE	Carbapenem resistant Enterobacterales
CRE-BSI	CRE blood stream infection
CREC	Carbapenem resistant <i>Escherichia coli</i>
CRKP	Carbapenem-resistant <i>Klebsiella pneumoniae</i>
CRPA	Carbapenem resistant <i>Pseudomonas aeruginosa</i>
CTX-M	Cefotaxime hydrolyzing β -lactamase-M
<i>cva/cvi</i>	Colicin V structural gene/ colicin V immunity gene
DAEC	Diffusely adherent <i>E. coli</i>
DDD	Defined daily doses
DDST	Double disk synergy test
DEC	Diarrhoeagenic <i>E. coli</i>
DHFR	Dihydrofolate reductase
DHP-I	Dehydropeptidase
DHPS	Dihydropteroate synthase
DIM-1	Dutch imipenemase

DOR	Doripenem
<i>E. coli</i>	<i>Escherichia coli</i>
<i>eae</i>	Intimin production gene
EAEC	Enteroaggregative <i>E. coli</i>
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic <i>E. coli</i>
EIEC	Enter invasive <i>E. coli</i>
EMB-	Eosin Methylene Blue
EPEC	Enteropathogenic <i>E. coli</i>
ESBL-EC	Extended-spectrum -lactamase (ESBL)-producing <i>E. coli</i>
ESBLs	Extended spectrum-lactamases
E-test-	Epsilometer test
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic <i>E. coli</i>
<i>fimA</i>	Type 1 fimbriae
<i>fimC</i>	Type 1 fimbriae (D-mannose-specific adhesin)
<i>fumC</i>	Fumarate hydratase
GES	Guiana extended spectrum
GIM	German imipenemase
GlcNAc	N-acetylglucosamine

<i>gyrB</i>	DNA gyrase
HAI	Hospital-acquired infections
HGT	Horizontal gene transfer
<i>hly</i>	Hemolysins gene
HUS	Hemolytic uraemic syndrome
HUSM	Hospital Universiti Sains Malaysia
<i>hlyA</i>	Haemolysin A
<i>icd</i>	Isocitrate/isopropyl malate
IMI-1	Imipenem-hydrolyzing beta-lactamase
IMP	Imipenem/Imipenemase
<i>irp2</i>	Iron regulatory protein
<i>iss</i>	Increased serum survival
<i>iutA</i>	aerobactin siderophore receptor
KPC	Klebsiella pneumoniae carbapenemase
LMICs	low- and middle-income countries
LPS	lipopolysaccharides
MAC	MacConkey
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MATE	Multidrug and toxic compound extrusion
MBL	Metallo-beta-lactamases
mCIM	Modified CIM
<i>mdh</i>	Malate dehydrogenase

MDR	Multi-drug resistant
MEM	Meropenem
MFS	Major facilitator superfamily
mg/L	Milligram per liter
MGEs	mobile genetic elements
MHA	Mueller-Hinton agar
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
min	minute
MLST	Multilocus sequence typing
MNEC	Meningitis-associated <i>E. coli</i>
<i>mprF</i>	Multiple peptide resistance factor
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MurNAcN	Acetylmuramic acid
MVAG	Malaysian Veterinary Antimicrobials Guidelines
MyAP-AMR	Malaysian Action Plan on Antimicrobial Resistance
NA	Nutrient agar
NBM	New born meningitis
NDARO	National Database of Antibiotic-Resistant Organisms
NDM	New Delhi metallo-beta-lactamase
NFW	Nuclease free water
NGS	Next generation sequencing
NmcA	Not metalloenzyme carbapenemase A

NPET	Nascent peptide exit tunnel
NSAR	National Surveillance of Antimicrobial Resistance
OMPs	Outer membrane proteins
ORFs	Open reading frames
OXA	Oxacillinases
PABA	Para-aminobenzoic acid
PACE	Proteobacterial antimicrobial compound efflux
PAIs	Pathogenicity islands
<i>papC</i>	Pilus associated with pyelonephritis
PATRIC	Pathosystems Resource Integration Center
PBP2a	Penicillin-binding protein 2a
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
<i>pho</i>	<i>Phosphate regulating gene</i>
PTC	<i>Peptidyl</i> transferase center
QC	Quality control
RNA	Ribonucleic acid
RND	resistance-nodulation-division
rRNA	Ribosomal RNA
<i>sat</i>	Secreted autotransporter toxin
SEPEC	Sepsis-associated <i>E. coli</i>
<i>sfa</i>	S fimbriae
SFC-1	<i>Serratia fonticola</i> carbapenemase-1

SIM	Seoul imipenemase
SME	Serratia marcescens enzyme
SMR	small multidrug resistance
SNPs	single-nucleotide polymorphisms
spp.	Species
ST	Sequence type
STEC	Shiga toxin-producing <i>E. coli</i>
<i>stx</i> ₁	Shiga toxin 1
<i>stx</i> ₂	Shiga toxin 2
TAT	Turn-around time
TBE	Tris Borate EDTA buffer
<i>tet</i>	Tetracycline resistance gene
<i>tetX</i> gene	flavin-dependent monooxygenase gene (tetracyclin resistance)
TMP	Sulphonamide and Trimethoprim
TolC	Outer membrane protein required for hemolysin secretion in <i>E. coli</i>
tRNA	Transfer RNA
TSB-	Tryptone Soy Broth
<i>tsh</i>	Temperature-sensitive haemagglutinin
TZB	Tazobactam
UPEC	Uropathogenic <i>E. coli</i>
UPGMA	Unweighted pair group method with arithmetic mean

UTIs	Urinary tract infections
VIM	Verona integron-encoded metallo-beta-lactamase
VRE	Vancomycin-resistant enterococci
WGS	Whole genome sequencing
WHO	World Health Organization
WOAH	World Organization for Animal Health
XDR	Extensive drug-resistant

LIST OF APPENDICES

- Appendix A Representative plates showing MIC test using E-test method. E-test strips impregnated with meropenem (MEM), ertapenem (ETP), doripenem (DOR), and imipenem (IMP) were used.
- Appendix B Genomic features of CREC shown by subsystems category distributions and counts
- Appendix C Antimicrobial resistance genes of CREC isolates as determined by the CARD (<https://card.mcmaster.ca/home>) with ‘perfect,’ ‘strict,’ and ‘loose’ hits.
- Appendix D SNP Calls of the CREC isolates show the number of positions that are shared and trusted between each isolate and the reference genome. *E. coli*_str.K-2.substr.MG1655 was used as a reference genome.
- Appendix E Prophage regions of CREC genomes show regions with intact (complete) questionable and incomplete prophages.
- Appendix F Multiple genome alignment of CREC isolates against the reference strain, *E. coli* str K.2 substr. MG1655. Each genome is laid out horizontally, and homologous segments are shown as colored blocks that are connected across genomes.
- Appendix G Pairwise alignment of CREC isolates against the reference strain, *E. coli* str K.2 substr. MG1655.

**PERBANDINGAN FENOMIK DAN GENOMIK *Escherichia coli* RINTANG
KARBAPENEM YANG DIISOLAT DARIPADA MANUSIA DAN AYAM**

ABSTRAK

Kemunculan Enterobacterales (CRE) rintang karbapenem adalah sangat membimbangkan dan kawalan penyebaran strain ini merupakan salah satu keutamaan yang ditetapkan oleh Pertubuhan Kesihatan Sedunia (WHO). Di Malaysia, laporan terkini menunjukkan peningkatan kes-kes CRE yang dilaporkan di hospital am dan hospital tertuari. Walau bagaimanapun; laporan kes CRE pada haiwan, terutamanya haiwan penghasil makanan seperti ayam dan itik di Malaysia adalah pada tahap minimum. Selain itu, sehingga kini tiada kajian yang melaporkan perbandingan CRE daripada manusia dan haiwan makanan di Malaysia. Oleh yang demikian, kajian ini dijalankan dengan objektif umum untuk membandingkan genomik *Escherichia coli* (CREC) tahan karbapenem yang dipencilkan daripada manusia dan ayam. Kajian ini dijalankan ke atas strain arkib pencilan klinikal CREC persumtif ($n=32$) dari Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian dan 384 sampel swab kloaka ayam yang diperolehi dari Pantai Timur Malaysia (Kelantan, Terengganu, dan Pahang). Pengenalpastian bakteria dilakukan melalui kaedah rutin bakteriologi dan diikuti dengan pencirian fenotip dan molekul serta penentuan epidemiologi molekul menggunakan penaipan jujukan berbilang lokus (MLST). Penjujukan keseluruhan genom (WGS) Illumina HiSeq™ berdaya tinggi dilakukan terhadap sepuluh isolat CREC terpilih bagi menentukan genomik perbandingan bagi pencilan CREC tersebut. Anotasi genom isolat tersebut kemudiannya dilakukan menggunakan peralatan RASTtk, BAKTA, dan eggNOG-Mapper, dan pengukuran kuantitatif dan kualitatif untuk analisis hiliran ad-hoc dijana menggunakan pelayan M1CR0B1AL1Z3R (Microbializer). Analisis WGS dilakukan menggunakan ResFinder

4.1, VirulenceFinder 2.0, alat SerotypeFinder 2.0, FimTyper versi 1.0 CHTyper 1.0, cgMLST 1.2, pMLST (2.0), CSI Phylogeny, MobileElementFinder, ISFinder Hunter 1.7, ISFinder 1.7, ISFinder. Analisis genom komprehensif tambahan dilakukan menggunakan beberapa kaedah analisis genomik yang berbeza. Keputusan menunjukkan kadar pengesanan CREC secara keseluruhannya adalah sebanyak 7.29% (28/384) iaitu 10.94% (28) daripada 256 *E. coli* yang diasingkan daripada swab kloaka ayam, yang ditentukan melalui kaedah pengesanan fenotip. Daripada kesemua isolat CREC, didapati 40% (24/60) isolat adalah daripada manusia dan ternakan ayam yang mempunyai lebih daripada satu gen karbapenemase termasuk gabungan gen-gen *bla_{NDM}+bla_{OXA-48}*, *bla_{NDM}+bla_{OXA-48}+bla_{IMP}* dan *bla_{OXA-48}+bla_{IMP}*. Penaipan molekul menggunakan kaedah MLST menunjukkan pengesanan ST69, ST131, ST155, ST405, dan ST410 yang telah diiktiraf sebagai keturunan pandemik berisiko tinggi. Analisis genomik perbandingan menunjukkan persamaan rapat di antara isolat CREC daripada manusia dan ayam yang terbukti daripada hampir semua profil genomik termasuk filogeni, pulau genomik, analisis SNP, plasmid, serotyping dan cgMLST serta profil genomik dan proteomik lain. Hasil analisis genomik perbandingan menunjukkan persamaan di antara strain CREC daripada manusia dan ayam yang sihat dan ini merupakan data epidemiologi penting berkaitan CREC dalam manusia dan ayam di Malaysia. Penemuan daripada kajian ini dapat membantu dalam pemahaman epidemiologi CREC tempatan dan kemungkinan berlakunya penyebaran dinamik CRE dalam konteks tempatan. Hasil penemuan kajian ini seterusnya dapat membantu dalam merangka strategi kawalan dan pencegahan berasaskan bukti yang secara langsung dapat menyumbang kepada program kawalan kerintangan antimikrobial kebangsaan untuk menjaga kesihatan awam.

COMPARATIVE PHENOMICS AND GENOMICS OF CARBAPENEM-RESISTANT *Escherichia coli* FROM HUMANS AND BROILER CHICKENS

ABSTRACT

The emergence of carbapenem-resistant Enterobacterales (CRE) has been alarming, and its control has been considered one of the priorities set by the World Health Organization (WHO). In Malaysia, recent reports show that the prevalence of CRE in general and tertiary hospitals has been alarmingly rising. However, little is known about the occurrence of CRE in animals, particularly food-producing animals such as broiler chickens in Malaysia. Moreover, there is no study on the comparative study of CRE from humans and food animals in Malaysia. Therefore, this study was conducted with the general objective of elucidating the comparative genomics of carbapenem-resistant *Escherichia coli* (CREC) from humans and broiler chickens. The study was conducted on clinical isolates archives of presumptive CREC isolates (n=32) from Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, and 384 cloacal swab samples of broiler chickens collected from East Coast Malaysia (Kelantan, Terengganu, and Pahang). Routine bacteriology followed by phenotypic and molecular characterization and determination of molecular epidemiology using multilocus sequence typing (MLST) were conducted. High-throughput Illumina HiSeqTM whole genome sequencing (WGS) of ten selected CREC isolates was done to determine the comparative genomics of the CREC isolates. The assembled genomes were annotated using RASTtk, BAKTA, and eggNOG- Mapper tools, and quantitative and qualitative measurements for *ad-hoc* downstream analyses were generated using M1CR0B1AL1Z3R server (Microbializer). Analyses of the

WGS were done using ResFinder 4.1, VirulenceFinder 2.0, SerotypeFinder 2.0 tool, FimTyper version 1.0 CHTyper 1.0, cgMLST 1.2, pMLST (2.0), CSI Phylogeny, MobileElementFinder, Alien Hunter 1.7, ISFinder and IslandCompare (v1.0). Additional comprehensive genome analyses were done using different genomic analysis pipelines. The results showed an overall CREC detection rate of 7.29% (28/384) which is 10.94% (28) of the 256 *E. coli* isolated from cloacal swabs of broiler chickens based on phenotypic detection methods. Out of all the CREC, 40% (24/60) of the CREC isolates from human and broiler chickens harbor more than one carbapenemase gene, including the combinations *bla*_{NDM}+*bla*_{OXA-48}, *bla*_{NDM}+*bla*_{OXA-48}+*bla*_{IMP}, and *bla*_{OXA-48}+*bla*_{IMP}. The molecular typing using MLST showed the detection of ST69, ST131, ST155, ST405, and ST410, which have been recognized as high-risk pandemic lineages. The comparative genomic analyses showed close similarities between CREC isolates from human and broiler chickens, which were evident from almost all the genomic profiles, including phylogeny, genomic islands, SNP analysis, plasmid, serotyping, and cgMLST and other genomic and proteome profiles. The comparative genomic analysis results showing similarities among CREC isolates from humans and apparently healthy chickens are important epidemiological data on CREC in human and broiler chickens in Malaysia. The findings from this study can help in better understanding the local CREC epidemiology and shed light on the possible CRE transmission dynamics in the local context. These findings, in turn, can help in devising of evidence-based control and prevention strategies that can contribute to the national antimicrobial resistance control programs to safe guard the public health.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The discovery of antimicrobials ushered in an era of hope that promised a medical revolution that enabled the treatment of several deadly diseases and immensely contributed to improved quality of life and life expectancy. Since the introduction of antibiotics on a large scale in the 1940s, deaths caused by infectious diseases have fallen by 70% (Plackett, 2021). Over a few decades, several potent antibiotics were discovered and further improved the effectiveness of antimicrobial therapy by availing more options for treating formerly untreatable infectious diseases. However, this same era also witnessed the fast emergence and spreading of antimicrobial resistance among several pathogens. Antibiotic-resistant bacteria continue to increase in diversity and potency, and new species and strains of bacteria have been emerging and spreading worldwide. This problem is exacerbated by the fact that pharmaceutical companies appear to have come to terms with apparent ‘surrender’ to the ever-evolving nature of resistant pathogens rendering every effort to develop new antibiotics futile (Plackett, 2020; Carlet *et al.*, 2014; WHO, 2014). The death toll due to infections caused by resistant bacteria is expected to rise from the current 700,000 to more than 10 million by 2050 (O’Neill, 2014). The annual economic impact of AMR was estimated to incur over US \$105 billion in losses worldwide, and developing countries, particularly Africa, are projected to suffer much of the relative economic impact with a 20% reduction in the region’s total economic output, which is equivalent to a reduction in GDP of US \$2895 billion by 2050 (Codjoe and

Donkor, 2017). Infection with resistant pathogens leads to severe sickness, prolonged admission, increased healthcare and second-line drug costs, and treatment failures. For example, in Europe, the loss due to AMR-related costs has been estimated to be more than nine billion euros annually. In addition, the Centers for Disease Control and Prevention (CDC) estimated that AMR incurs an additional USD \$ 20 billion in direct healthcare costs and USD \$ 35 billion in indirect losses due to loss of productivity every year in the United States (Dadgostar *et al.*, 2019).

The ever-increasing emergence and spread of AMR are attributed to several factors, including overpopulation, increase in global migration, imprudent and increased use of antibiotics in humans and animals, selection pressure of antibiotics on microorganisms, environmental changes, wildlife spread, poor sanitation, and lack of appropriate disposal of sewerage are among the significant contributors to the (Aslam *et al.*, 2018). Among the several causes of AMR, the excessive use of antibiotics has been identified as a major driving factor in the evolution and spread of resistant bacteria. This has been demonstrated by epidemiological studies showing the correlation between antibiotic usage and the development of resistance in bacteria (Cock and Cuny, 2020; Read and Woods, 2014). In bacteria, resistance genes can be intrinsically presented or acquired from other bacteria through mobile genetic elements (MGEs) such as plasmids, insertion sequences, and transposons (Read and Woods, 2014). Apart from HGT, bacteria may also develop resistance spontaneously through mutation. The use of antibiotics creates selective pressure and removes drug-sensitive competitors favoring the survival of resistant bacteria due to natural selection (Read and Woods, 2014). Despite warnings regarding overuse, antibiotics are overprescribed worldwide (Ventola, 2015).

Antimicrobial resistance is a global threat to public health, animal health and production, food safety and food security, and environmental health. Multi-drug resistant (MDR) bacteria or “superbugs” continue to exist, emerge, and spread along the human-animal-environment interface with intertwined dynamics of sharing of resistance determinants among these triads. The common causes of AMR include overuse and misuse of antibiotics in humans and animals accompanied by poorly controlled antibiotics trading, increased international travel, poor sanitation and hygiene, and release of non-metabolized antibiotics and their residues into the environment through manure/feces. These factors facilitate the genetic selection pressure, which favors the emergence of infections caused by MDR bacteria in the community (van Boeckel *et al.*, 2015). Antibiotics are also used in food-producing animals such as poultry, cattle, and pigs, and it is projected that an increase of up to 67% in such antibiotic uses will be recorded in highly populated countries (van Boeckel *et al.*, 2015). Due to varying degrees of host specificities and the complexity of transmission, it is difficult to quantify the spread of resistant bacteria between humans and animals (Cock and Cuny, 2020; Muloi *et al.*, 2018; Van Boeckel *et al.*, 2015). However, initiatives taken to reduce antimicrobial usage in animals have shown some promise in reducing the occurrence of resistant bacteria in humans and animals (Stoica and Cox, 2021).

The global increase in demand for animal protein has become a worldwide phenomenon and dietary trend, notably in developing countries. Although meat production in high-income countries has plateaued since 2000, growth rates of 40%, 64%, and 68% were recorded in South America, Africa, and Asia, respectively. This growth and increased demand for animal protein in low- and middle-income countries (LMICs) have been enabled by the global expansion of improved animal production systems which

heavily rely on antimicrobials to maintain and enhance food animal health and production. Records show that up to 73% of all antimicrobials sold globally are used for food animal production. This scenario has been accompanied by a growing body of evidences showing the link between extensive antimicrobial use in food animal production and the rise of infections caused by antimicrobial-resistant pathogens both in animals and humans (Pokharel *et al.*, 2020).

In recent years, ample evidence has shown that the public health challenges caused by AMR are increasing, and coordinated global interventions are required to contain these ever-increasing threats. The public health and economic burdens of AMR have been showing increasing trends, and worldwide data show that common and diverse bacterial pathogens have alarmingly become resistant to currently available antimicrobials (Codjoe and Donkor, 2018). To counter the rising threats of AMR, the World Health Organization (WHO) developed a ranking list of antimicrobial-resistant pathogens that can be used as a guide for determining areas of focus and effective resource allocation (WHO, 2017). Carbapenem resistance by Enterobacterales was identified as one of the pathogens that have been assigned a high critical priority.

Carbapenems are a group of antibiotics that have been used as a lifesaving and last-resort antibiotic to treat infections caused by MDR bacteria. Carbapenem-resistant Enterobacterales are among the major challenges to healthcare systems. Due to limited antimicrobials, infections caused by CRE are more challenging to treat and are commonly associated with high mortality and morbidity (Dong *et al.*, 2020). Therefore, the ongoing increase of CRE prevalence in Enterobacterales species commonly associated with severe infections in healthcare settings is a matter of major concern. The development and spread of carbapenem resistance are mostly attributed to CRE and are mostly driven by the

emergence and spread of carbapenemases which are specific group carbapenem hydrolyzing beta-lactamases. Most carbapenemases-producing Gram-negative bacteria are resistant to carbapenems or are less susceptible to the same antimicrobials and other broad-spectrum agents (Iovleva *et al.*, 2017).

In Malaysia, the carbapenem resistance rate in *E. coli* is still less than 1%, and a fluctuation in imipenem resistance was seen during the three-year periods (2018-2020) with prevalence rates of 0.8%, 0.5%, and 0.7% being recorded in 2018, 2019, and 2020 respectively. In comparison, the prevalence of meropenem-resistant *E. coli* increased from 0.6% in 2019 to 0.7% in 2020. Likewise, the prevalence of imipenem and meropenem-resistant *Klebsiella pneumoniae* (*K. pneumoniae*) also showed an increasing trend, with prevalence rates of 1.7% and 2.1% in 2019 to 2.4% and 2.8%, respectively, in 2020 (NSAR, 2020). According to ten-year (2006 to 2017) data compiled by the National Surveillance of Antimicrobial Resistance (NSAR) of Malaysia indicated that the overall prevalence of CREC declined from 0.5% in 2010 to 0.2% in 2014 (Hsu *et al.*, 2017). However, a more recent report on the prevalence of CRE in a tertiary hospital in Malaysia shows that the prevalence of CRE in 2015 and 2016 was 0.3% (5/1590) and 1.2% (17/1402), respectively. The same study reported that the majority (81.8%) of the isolates were *Klebsiella pneumoniae*, followed by *Serratia marcescens*, *E. coli*, and *Citrobacter koseri* (Mohamed *et al.*, 2018). However, the data on the prevalence of CREC in animals in Malaysia is scarce. Since the inception of this study, a single preliminary study by Ghazali *et al.* (2020) reported a CRCE prevalence of 1% (2/200) from antemortem cloacal swab samples collected from broiler chicken from an abattoir in Terengganu. However, this study was limited in scope and depth of investigation.

1.2 Justification of the study

Reports on the occurrence and prevalence of CRE in Malaysia showed that there is an alarmingly increasing trend in the incidences of CRE-related infections in general and tertiary hospitals in Malaysia (Zaidah *et al.* (2017)). This may imply that the status of CRE in Malaysia is still not fully investigated, and the prevalence, diversity, resistance patterns, and different potential sources of CRE have not been sufficiently investigated. In addition, almost all the reported CRE prevalence studies in the country have been conducted in hospital settings, and the occurrence and characteristics of CREC from animals, particularly CREC from food animals, have not been well investigated. There is also a paucity of data on the occurrence of CREC and whether broiler chickens may serve as CREC that may spread to humans and possibly cause infections in Malaysia. In addition, there is no data on the comparative genomics of CREC isolates from humans and broiler chickens in Malaysia. Understanding the antimicrobial resistance patterns, virulence profiles, molecular epidemiology, and genomic characteristics of CREC from human and broiler chickens will provide a detailed and better insight into the existing status of these pathogens and may help complement the national AMR control strategy.

1.3 Research questions

1. How prevalent is CREC in broiler chickens in East coast Malaysia (Kelantan, Terengganu, and Pahang)?
2. What are the antimicrobial resistance, virulence, and phylogenetic characteristic of CREC isolates from humans and broiler chickens?

3. What is the molecular epidemiology of CREC isolates from human and broiler chickens, and how the local CREC strains are related to the globally disseminated *E. coli* strains?
4. What is the comparative genomics of CREC isolated from humans, and how does this comparative study help in generating epidemiologically useful insights?

1.4 Objectives of the study

1.4.1 General objectives

1. This study aimed to investigate the comparative genomics of carbapenem-resistant *E. coli* (CREC) from humans and broiler chickens in East Coast Malaysia (Kelantan, Terengganu, and Pahang)

1.4.2 Specific objectives

2. To determine the prevalence of CREC in broiler chickens in East coast Malaysia (Kelantan, Terengganu, and Pahang)
3. To determine antimicrobial resistance patterns of carbapenem-resistant *E. coli* isolates from broiler chickens and humans.
4. To conduct molecular characterization of CREC isolates based on the detection of carbapenemases genes, virulence genes, and phylogenetic characteristics.
5. To determine the molecular epidemiology of carbapenem-resistant *E. coli* isolates
6. To elucidate the comparative genomics of carbapenem-resistant *E. coli* isolates from human and broiler chickens.

CHAPTER TWO

LITERATURE REVIEW

2.1 General characteristics of *Escherichia coli*

Escherichia coli is a member of the *Enterobacterales* and is characterized as a short, non-spore-forming, facultatively anaerobic Gram-negative bacillus. It readily grows on ordinary media without the need for special enrichment. Biochemically, it is characterized by indole production, absence of citrate fermentation, positive reaction on methyl red test, and negative Voges–Proskauer reaction with no urease production (Bhutia *et al.*, 2021). *Escherichia coli* is a predominant aerobic commensal and a member of gut microbiome of vertebrates. The majority of *E. coli* strains are commensals of the intestinal tract of warm-blooded animals and humans. In general, commensal *E. coli* are harmless and symbiotically live within the host while causing infections rarely in immune-competent hosts (Ramos *et al.*, 2020). The bacteria is present in almost 90% of humans and is commonly found at a concentration of 10⁷ to 10⁹ colony-forming units (CFU) per gram of feces. *Escherichia coli* is also an opportunistic pathogen with high pathogenic potential to cause intestinal and extraintestinal infections (Denamur *et al.*, 2021).

Pathogenic and non-pathogenic strains of *E. coli* are differentiated depending on their acquisition of virulence factors or loss of functional genes encoding adhesion, invasion, colonization, cell surface molecules, secretions, transport, survival, and iron metabolism (Sora *et al.*, 2021). In terms of their genomes, *E. coli* strains may have varying numbers of genes ranging from 4,000 to 5,000. Among these, about 3,000 genes are present in different *E. coli* strains, while the rest of the genes are mostly associated with the genes that are responsible for colonization or virulence. The use of advanced genomic

tools such as next-generation sequencing (NGS) has enabled a clearer understanding of the plasticity of *E. coli* genomes by revealing much of the core and accessory genomes of both commensal and pathogenic *E. coli* strains (Poirel *et al.*, 2018).

2.2 Pathogenic *Escherichia coli*

Escherichia coli strains cause several extraintestinal pathologies, including various intra-abdominal, pulmonary, soft tissue, skin, and urinary tract infections, new born meningitis (NBM), and bacteremia. The major intestinal infections caused by *E. coli* include different forms of diarrhea, including hemolytic and uraemic syndrome (HUS). Infections such as urinary tract infections (UTIs), renal failure in HUS in children, and neurologic complications in NBM are often associated with high mortality and morbidity.

In recent years, the incidences of extraintestinal infections caused by *E. coli* have been increasing, and HUS epidemics, such as the 2011 epidemic in Europe, have become more common. This problem has been further aggravated by the rising incidence and spread of antibiotic-resistant *E. coli*, making this pathogen the third-ranked ‘priority pathogen’ among the 12 antibiotic-resistant pathogens listed by the WHO (Denamur *et al.*, 2021). In animals, *E. coli* is one of the major causes of diarrhea, along with other pathogens such as rotavirus, coronavirus, *Cryptosporidium parvum*, or a combination of these pathogens (Poirel *et al.*, 2018). The bacterium can also cause UTIs in small animals (Teh, 2022).

In chickens, *E. coli* infections cause several disease syndromes, including septicemia, enteritis, omphalitis, respiratory tract infection, swollen head, and cellulitis (Swelum *et al.*, 2021). Pathogenic *E. coli* strains cause various diseases through multiple

mechanisms of pathogenesis, including the colonization of the mucosae, host immune evasion, replication, and tissue injury.

Pathogenic *E. coli* are classified into pathotypes based on pathogenicity mechanisms (patterns of attachment and invasion), virulence (toxin production, presence or absence of virulence plasmids, mechanisms of attachment), and the clinical syndromes they cause (Kaper *et al.* 2004). These pathotypes are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), including Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Pakbin *et al.*, 2021). The different *E. coli* pathotypes and their characteristics are summarised in table 2.1.

2.3 Antibiotics Discovery and the ensuing medical Revolution

Antibiotics are chemical substances that are secreted by microorganisms or synthetic products with bacteriostatic or bactericidal properties and are used to inhibit the growth of or kill pathogenic bacteria (Bhattarai *et al.*, 2020; Pancu *et al.*, 2021). Throughout the ages, infectious diseases have been challenging human existence and quality of life. One of the marked historical pieces of evidence of the cataclysmic effects of infectious diseases was the emergence and spread of the Bubonic plague, which claimed the lives of approximately one-third of Europe's population between 1347 and 1350. In general, infectious diseases remained the leading causes of death up to the early 1900 (Ribeiro da Cunha *et al.*, 2019).

Table 2.1. *Escherichia coli* pathotypes and their main characteristics.

Pathotype	Identification criteria	Affected host (s)	Common Diseases and Symptoms	Major virulence genes	Phylogroups	Common sequence types (STs)	References
EPEC	Adheres to the intestinal epithelium and effaces microvilli.	Humans, Domestic animals, chicken	Diarrhea in children, Watery diarrhea and vomiting	Bfp, Intimin, LEE	B2, C, D, F	ST131, ST88, ST69, ST62	Denamur <i>et al.</i> (2020) Kaper <i>et al.</i> (2004)
EHEC/STEC	Shiga toxin production (Presence of <i>stx</i> genes)	Human, Cattle, Sheep	Hemorrhagic colitis, HUS, Bloody diarrhea	<i>stx</i> , <i>eae</i> , <i>ehxA</i>	B1, E	ST11 ST29 ST17	Garcia and Fox (2021)
ETEC	Production of adhesins enterotoxins	Human Pig Cattle Sheep	Traveler's diarrhea, Watery diarrhea and vomiting	LT, STa, STb, EasT, F4	A, B1, C, E	Multiple	Dubreuil <i>et al.</i> (2016)
EAEC	Aggregative adhesion on enterocytes	Human, domestic mammals	Diarrhea in children, Diarrhea with mucus and vomiting	Aggregative adherence fimbriae (<i>aaf/agg</i>) and	aatA	Multiple	Riley (2020)
EIEC	Colonocyte invasion	Human	Shigellosis-like, Watery diarrhea; dysentery	<i>ipaC</i> , <i>ipaH</i> , <i>isc</i> , var, Shiga toxin, hemolysin, Cellular invasion, <i>Ipa</i>	A, B1, E	ST6 ST270 ST280	Denamur <i>et al.</i> (2020), Garcia and Fox (2021)
DAEC	Diffuse adhesion on enterocytes	Human	Acute diarrhea in children, Watery diarrhea, recurring UTI,	Adhesion encoding genes (<i>afa</i> & <i>dra</i>), <i>Daa</i> , <i>AIDA</i>	All phylogroups	Multiple	
ExPEC	Extra-intestinal infection	Human, domestic mammals, poultry	Various extra-intestinal infections	Adhesion encoding genes, toxins, protectins & iron capture systems	B2, C, D, F	ST131, ST69, ST88, ST62	Allocati <i>et al.</i> (2013), Denamur <i>et al.</i> (2020),
ExPEC (APEC)	Isolated from birds	Poultry Human	Collibacillosis	pColV genes, Type 1 and P fimbriae; K1 capsule	B2, C	ST95, ST88	Denamur <i>et al.</i> (2020), Allocati <i>et al.</i> (2013)

The discovery and use of antibiotics radically impacted human health and quality of life to the extent that it was considered a ‘medical miracle’ of the 20th century. The years 1930–1962, which is often called the golden age of antimicrobial discoveries, saw the rapid invention and development of 20 different classes of novel antimicrobials, many

of which contributed to the betterment of human health and wellbeing for more than six decades (Dhingra *et al.*, 2020). The ‘miraculous’ effects of antibiotics were later tapped for non-therapeutic applications, including their use in enhancing agricultural productivity, particularly as growth promoters to increase food production (Manyi-Loh *et al.*, 2018).

The golden age of antibiotics was marked by overwhelming success in treating many life-threatening infectious diseases. This resulted in the overly optimistic view that diseases caused by microorganisms would finally be conquered in a very short period owing to the rapid antibiotics discoveries which were believed to enable the control of infectious diseases as a public health problem (Aminov, 2010). A notable remark of triumph on infectious diseases with the help of the apparently indomitable antibiotics was made by the US Surgeon General in 1970, who was said to opine that ‘it was time to close the book’ on infectious diseases and redirect national resources to the control and prevention of chronic problems such as cancer and heart disease (WHO, 2018). However, that was not meant to materialize as all the euphoric optimisms about antibiotics ‘miracles’ soon began to be shredded as resistant bacteria emerged. Even then, apparently, no one could have fully grasped the microbial resolve and capabilities enabling the pathogens to attain the status of apparent invincibility attributed to their ability to resist multiple antimicrobials, including resistance to the most potent antibiotics.

2.4 Mechanisms of Action of Antibiotics

At therapeutic concentrations, antibiotics are sufficiently potent to be effective against infection while simultaneously presenting minimal toxicity to the patient. Based on their action on bacteria, they are categorized as bactericidal or bacteriostatic. Natural

antibiotics originate from different species of bacteria and fungi as secondary metabolic products. As these substances are not essential for bacterial cell survival, they are usually produced in demand. Usually, bacteria produce these antibiotics against other competing bacteria and persist in challenging environmental conditions. In general, antibiotics that occur in their natural forms are less potent and have fewer side effects compared to synthetic antibiotics. Common natural antibiotics include penicillin, streptomycin, gramicidin, and chlortetracycline. Synthetic antibiotics are produced in the laboratory and approved for clinical use. Common examples of synthetic antibiotics include cephalosporin C, fluorocyclines, linezolid, and meropenem. Compared to natural antibiotics, synthetic antibiotics act faster and have higher toxicity to pathogens (Upmanyu and Malviya, 2020). The selective toxicity of these substances induces bactericidal and bacteriostatic to the bacterial cells with minimum side effects to the patients. The selective blocking of critical bacterial metabolic pathways disrupts bacterial cell structures (Abushaheen *et al.*, 2020; Walsh, 2004). The different mechanisms of actions of groups of antimicrobial agents by which they kill or inhibit bacteria are discussed in the following sections.

2.4.1 Interfering with cell wall synthesis

The cell wall of a bacterial is the outermost elastic structure that maintains the bacterial cell structural integrity by protecting the bacteria from adverse osmotic effects that may cause bacterial cell disintegration. The peptidoglycan layer is made of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) long glycan chains cross-linked by Penicillin Binding Proteins (PBP). This bacterial structure is the main target for β -lactam and glycopeptides antibiotics (Nikolaidis *et al.*, 2014). By acting on

the peptidoglycan layer of the bacterial cell wall, these antibiotics bacterial cell wall synthesis and disrupt the cell wall structure, thereby inducing bacterial cell lysis. Such antibiotics include glycopeptides (vancomycin and teicoplanin) and β -lactams (carbapenems, penicillins, cephalosporins, and monobactams).

The β -lactam antibiotics are a wide class of antibiotics produced by the fungus *Penicillium* and were discovered in the 1930s. These antibiotics are characterized by the presence of an azetidinone nucleus containing the carbonyl β -lactam, which is essential for the activity (Kapoor *et al.*, 2017). There are several classes of β -lactam antibiotics that target the penicillin-binding proteins (PBPs) and are used against different bacterial species. The β -lactam antibiotics are structurally similar to the D-Ala-D-Ala dipeptide of the developing peptidoglycan to which it covalently binds at the serine active binding site on the PBPs blocking the formation of linkage between the peptidoglycan layer, which ultimately blocks cell wall synthesis (Zapun *et al.*, 2008). The β -lactams are the most popular bactericidal antibiotics the most common and popular antibiotics used to treat several bacterial infections and usually have lower toxicity with the exception of allergic reactions in sensitive individuals (Balsalobre *et al.*, 2020).

2.4.2 Inhibition of protein synthesis

Protein synthesis is a complex and essential biological process in living cells that occurs through processes including transcription and translation, which are carried out through initiation, elongation, termination, and recycling. The differences in structures of the bacterial ribosome and the ribosome of eukaryotes enable the selective inhibition of bacterial protein synthesis. The antibiotics achieve the inhibition by blocking the protein synthesis process at the 30S or 50S subunits of the 70S bacterial ribosome. By inhibiting

protein synthesis, the antibiotics stop or retard bacterial cell growth (Tenover, 2006). Common examples of 30S subunit-blocking antibiotics are Macrolides, aminoglycosides, and tetracycline. The positively charged carbohydrate groups of the antibiotics bind to the negatively-charged plasma membrane and diffuse into the bacterial cell. Once inside the bacteria cell, they attach to the 30S subunit of the ribosome at the A-site, reversing the process into extra-helical translation, which causes the formation of a faulty mRNA-tRNA pairing that leads to errors in translation and protein synthesis (Wilson, 2014; Garneau-Tsodikova and Labby, 2016). Whereas the 50S ribosome subunit of the bacterial cell forms a polypeptide chain in the peptidyl transferase center (PTC). Moreover, the 50S subunit contains a nascent peptide exit tunnel (NPET) which serves as a gate to the polypeptide chain leaving the ribosome. A common example of 50S subunit inhibiting antibiotics is chloramphenicol which acts by binding to the 50S subunit between the NPET and the PTC, which prevents the incorporation of newly made polypeptides cross the channel. This leads to disruption in the elongation step and results in the inhibition of bacterial protein synthesis (Ban *et al.*, 2000).

2.4.3 Inhibition of nucleic acid synthesis

The synthesis of bacterial DNA requires topoisomerases; the lack of these enzymes leads to the formation of abnormal DNA (Abushaheen *et al.*, 2020; Pommier *et al.*, 2010). For example, fluoroquinolones function by inhibiting the enzyme DNA gyrase enzyme in Gram-negative bacteria, which is vital in initiating bacterial DNA replication. They also inhibit the enzyme topoisomerase IV, which is critical for the daughter-cell segregation in Gram-positive bacteria. The quinolones bind to topoisomerase IV or II and impede bacterial DNA synthesis by modifying the supercoiling of DNA, causing the

interruption of double-stranded bacterial DNA and leading to the death of the bacteria. The antibiotic effect is achieved through the pathway that may or may not depend on protein synthesis pathways (Abushaheen *et al.*, 2020).

2.4.4 Inhibition of metabolic pathways/bacterial enzymes

The metabolic processes and synthesis of various cellular components of prokaryotic and eukaryotic cells require reduced folate co-factors as a vital component. In eukaryotic cells, the uptake of folate occurs through an active transport system, whereas in prokaryotic cells acquire folate through the de novo synthesis pathway. This makes the folate biosynthesis pathway a viable target for antibiotics (Bertacine Dias *et al.*, 2018). The folate synthesis pathway utilizes the enzyme dihydropteroate synthase (DHPS), which requires para-aminobenzoic acid (PABA). Sulphonamides act by inhibiting PABA in bacterial folate synthesis. The fact that sulphonamides share structural similarity with PABA makes it a competitive inhibitor to which folates can bind as alternatives which in turn deprives the bacteria cell of the vital nutrient, thereby leading to inhibition of the bacterial cell growth. Diaminopyrimidine antibiotics (i.e., Trimethoprim) inhibit dihydrofolate reductase (DHFR), which is the last enzyme in the folate biosynthesis pathway (Schober *et al.*, 2019). By incorporating it into the precursors, sulfonamides block the formation of folic acid and form a reactive and antibacterial pseudometabolite. Sulfonamides are bacteriostatic antibiotics with antifungal and antimalarial properties. The combination of sulphonamide and diaminopyrimidine antibiotics has been used in the treatment of several infectious diseases, including urinary tract infections. However, the emergence of remittance against these antibiotics combination proved to be a challenge (Giles *et al.*, 2019). Despite their common side effects, sulfonamides are considered to be

among the most effective and safe antibiotics in WHO's list of essential medicine (WHO, 2015).

2.4.5 Interruption of bacterial membrane

The bacterial membrane plays a vital role in ensuring bacterial cell survival and thus can be a good target for antimicrobial agents. Unlike Gram-positive bacteria, Gram-negative bacteria have an added protective layer on the outer membrane composed of lipopolysaccharides (LPS). Several potent antimicrobial agents hinder the formation of mature LPS by inhibiting LPS synthesis at various stages. As a result, the bacteria become more prone to imbalances in osmotic pressure due to the increasing permeability that leads to bacterial cell destruction (Epand *et al.*, 2016). Polymyxins which are considered the last-line antibiotics for the treatment of infections caused by MDR Gram-negative bacteria, are common examples of bacterial cell membrane synthesis inhibiting antibiotics. The bacteriostatic action of Polymyxins is achieved through the interaction between polymyxin, a positive charge, with the lipid A of LPS, which is negatively charged. This binding alters the bacterial structure and makes the cell membrane extra permeable. This, in turn, leads to disruptions in osmotic pressures in the bacterial cell leading to the outflow of cellular components, inhibition of respiration, and surge of water inflow, which finally leads to lysis and death of the bacterial cell (Yin *et al.*, 2020). Figure 2.1 shows the summary of the major mechanisms of antimicrobial actions and their corresponding potential mechanisms of resistance.

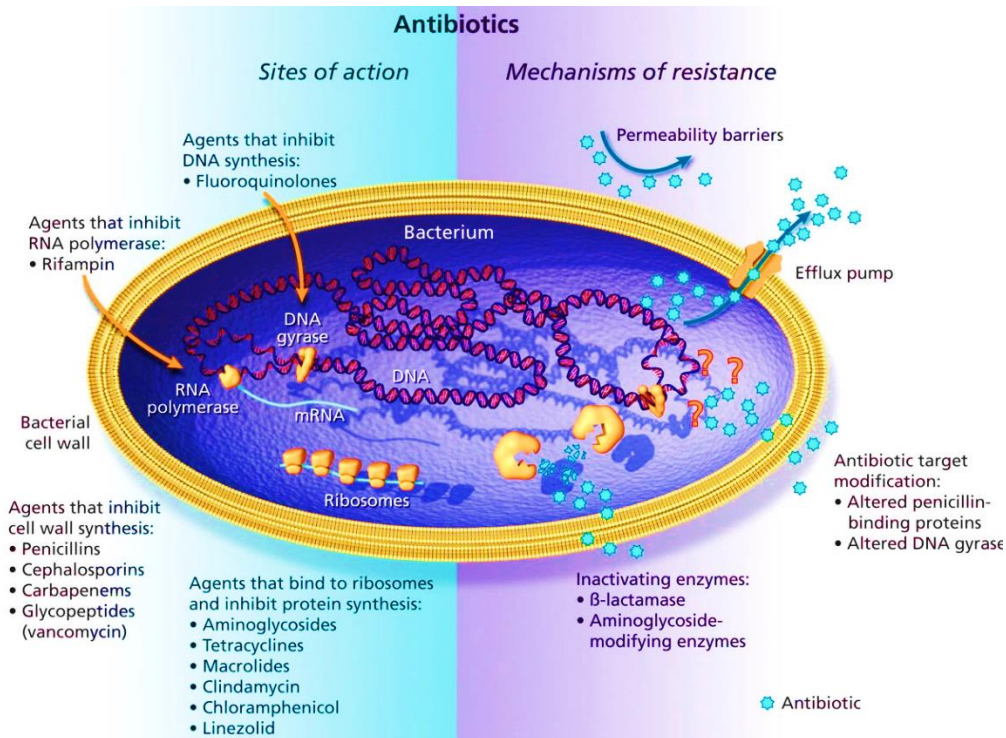


Figure 2.1. Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents. Adapted from (Mulvey and Simor, 2009)

2.5 Antimicrobial Resistance

Antimicrobial Resistance occurs when pathogens such as bacteria, viruses, fungi, and parasites evolve through time and no longer respond to antimicrobial therapy, thereby making it increasingly difficult or impossible to treat infections and increasing the severity of illness, transmission, and death (WHO, 2021). Antimicrobial resistance has been an acknowledged fact since the dawn of the antibiotic era. In fact, it did not take long for resistant bacteria to emerge soon after the discovery of antibiotics and their introduction into clinical use. However, the threats of AMR became of serious concern only in recent decades following the emergence of diverse and dangerous resistant strains, which continues to occur at an alarming rate. This escalating evolution of resistance by bacteria

coupled with an apparently exhausted and lessened antibiotic pipeline has somehow caused the fear that the ushering of a post-antibiotic era is apparently eminent (Fair and Tor, 2014, Jackson *et al.*, 2018). Antibiotic resistance is a global public health threat that affects individuals, communities, societies, and countries all over the world. The impacts of AMR are not only limited to health care but also to veterinary and agricultural sectors. Resistant bacteria have been evolving and increasingly posing enhanced resistance levels characterized by higher frequency and strength of resistance against all antibiotics that have been approved for clinical use worldwide. This has led to the shortening of the clinical usability life span of newly approved antibiotics to less than ten years before high incidences of resistance demand the guarded usage of these antibiotics (Spagnolo *et al.*, 2021). The emergence of AMR is a multifaceted and complex issue that has been compounded by several contributing factors. Although the common notion assumes that AMR emerged as a result of the introduction of antibiotic usage in health care and other sectors, evidence suggests otherwise and that resistant bacteria existed well before the discovery and clinical use of antibiotics. This can be illustrated by the fact that penicillin-resistant *Staphylococcus* species were identified even before the discovery, industrial production, and widespread clinical usage of the first antibiotic in 1943 (Hwang and Gums, 2016). This observation is explained by the fact that the genetic diversity required for the development of penicillin resistance in *Staphylococcus* could not have developed in the shorter time frame following the introduction of penicillin in clinical use. Rather it implies that bacteria do have an intrinsic resistance encoded in their genome that has been evolving over centuries. Thus, the development of antimicrobial resistance in bacteria is a natural process that occurs with or without human intervention (Fair and Tor, 2014). However, the introduction of penicillin in clinical use was believed to create selective

pressure on bacteria which induced bacterial adaptive mechanisms that enabled the acceleration of natural selection and the emergence of more resistant or more virulent bacteria. This problem was further aggravated by the widespread introduction of multiple potent antimicrobials and their imprudent use in humans, animals, and agriculture (Hwang and Gums, 2016; Fair and Tor, 2014).

2.5.1 Mechanisms of antimicrobial resistance

As part of their evolution processes that took place over millions of years, diverse species of bacteria developed complex and sophisticated mechanisms of survival in the presence of antimicrobial molecules. Such an interesting sophistication in bacterial cell resistance is their ability to resist a particular class of antibiotics through multiple biochemical pathways, which helps the bacteria to have a tool kit of mechanisms to evade the effect of antibiotics (Munita and Arias, 2016). In general, AMR mechanisms are broadly classified into intrinsic (natural) and acquired resistance. Intrinsic or natural resistance refers to the inherent nature of bacterial species to be resistant to some antibiotics due to their unique structural/functional characteristics. Whereas acquired resistance is the development of antibiotic resistance by naturally susceptible bacteria through the acquisition of specific genetic codes from other bacteria (Abushaheen *et al.*, 2020).

2.5.1(a) Genetic Basis of Antimicrobial Resistance

Endowed with higher genetic plasticity, bacteria are capable of responding to arrays of threats from their environments, including the presence of antimicrobial

molecules that may threaten their existence. Because they share the same ecological niches with antimicrobial-producing microorganisms, different species of bacteria have evolved primeval mechanisms to thwart the potentially deadly effects of antibiotic molecules to ensure their survival. The two major genetic strategies used by bacteria that are of evolutionary importance to survive and thrive in the presence of antimicrobial substances are through mutations in gene(s) that are often associated with the mechanism by which the antimicrobial compounds act on the bacteria and through the acquisition of AMR encoding foreign DNA through HGT (Munita and Arias, 2016; Peterson and Kaur, 2018).

2.5.1(b) Mutational Resistance

Because of the diverse and intricate mechanisms of mutation, antibiotic resistance acquired through mutational changes are diverse and complex. In general, bacterial cell mutation resulting in the development of antimicrobial resistance interferes with the action of antibiotics through one of the following mechanisms, *i*) antimicrobial target modifications, *i*) reduced drug uptake, *ii*) activation of efflux mechanisms, *iv*) global alteration of vital metabolic pathways through modulation of regulatory networks (Lopatkin *et al.*, 2021; Munita and Arias, 2016). Specific examples of antimicrobial resistance development through mutational changes will be discussed in the following sections.

2.5.1(c) Horizontal Gene Transfer

Acquisition and incorporation of foreign DNA material from other bacteria or environments through HGT are vital driving factors that enormously contribute to the

evolution of bacterial pathogens and their ability to develop AMR (Munita and Arias, 2016). Most of the antimicrobials in clinical use are derived from natural environmental products, including soil. As stated earlier, bacteria living in environments with natural antimicrobials harbor intrinsic genetic determinants of resistance, and there is ample evidence suggesting that environmental resistome is a robust source of antimicrobial resistance acquisition by pathogenic bacteria. The genetic exchange of resistance genes has been frequently implicated as the cause of AMR emergence and dissemination. Naturally, three main strategies are used by bacteria to acquire external genetic material, i) conjugation (bacterial “sex”), ii) transduction (phage mediated), and transformation iii) (incorporation of naked DNA).

Transformation is considered the simplest type of HGT; however, under natural circumstances, only very few bacteria with clinical relevance are able to incorporate naked DNA and develop resistance. Nosocomial emergence of resistance is often attributed to conjugation, which is a more efficient gene transfer method through cell-to-cell contact between the donor and recipient bacteria. It has been shown that conjugation is more likely to occur at high rates in the gastrointestinal tract of humans receiving antibiotic treatment (Niel *et al.*, 2021). Although the direct transfer of resistance genes from chromosome to chromosome is possible, conjugation mostly occurs through the movement of MGEs. Among the MGEs, plasmids and transposons play important roles in the emergence and dissemination of AMR among clinically important bacteria (Munita and Arias, 2016; Partridge *et al.*, 2018). Lastly, integrons are ancient structures that promote bacterial evolution through the acquisition, storing, disposing, and resorting of reading frames in mobile gene cassettes. They are considered one of the most efficient mechanisms for accumulating AMR genes. Integrons are one of the main drivers of AMR as they provide

an efficient yet simple mechanism for the incorporation of new genes into bacterial chromosomes, maintenance of the functional equipment, and a robust strategy of the genetic interchange (Sabbagh *et al.*, 2021).

2.5.1(d) Mechanistic Bases of Antimicrobial Resistance

Based on the biochemical route involved in resistance, antibiotic resistance mechanisms can be categorized as follows: (i) drug uptake limitation, (ii) drug target modification, (iii) drug inactivation; and (iv) drug efflux (figure 2.2). Because of their structural differences and others, all four mechanisms are used by Gram-negative whereas since Gram-positive bacteria lack the lipopolysaccharide in the outer membrane, they are less likely to use limiting the uptake of a drug and drug efflux mechanisms (Reygaert, 2018; Uddin *et al.*, 2021). Each of these resistance mechanisms has its own specific biochemical pathways that will be further elaborated in the following section.

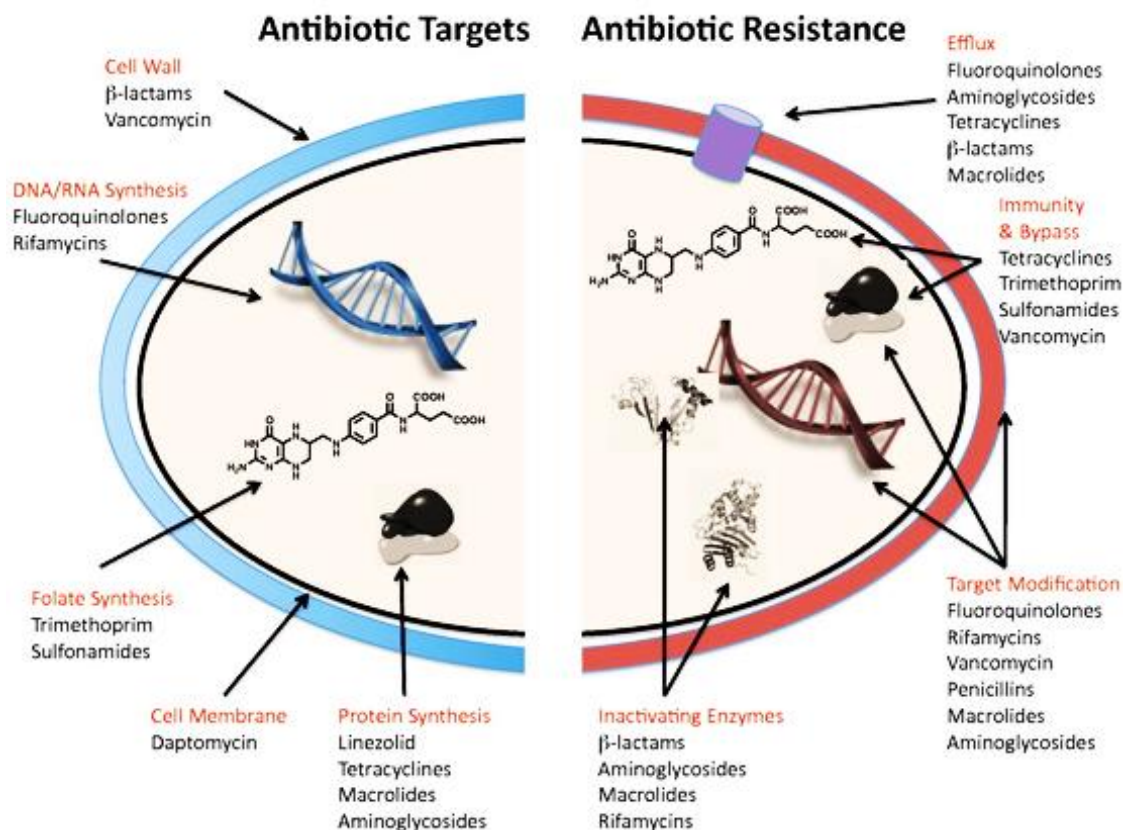


Figure 2.2. Antibiotic targets and mechanisms of resistance. Adapted from (Wright (2010))

2.5.1(e) Drug uptake limitation

Naturally, bacterial species differ in their abilities to limit the uptake of antimicrobial agents. For example, the structural and functional features of the Lipopolysaccharides (LPS) layer in Gram-negative bacteria serves as a barrier to certain types of molecules, arming the bacteria with intrinsic resistance to several groups of antibiotics (Bertani and Ruiz, 2018). It is due to this structural difference that glycopeptide antibiotics such as vancomycin are incapable of penetrating the outer membrane barrier and are, therefore, not effective against Gram-negative bacteria. The net change in permeability across the cell membrane barrier is determined by the hydrophobicity of the antibiotics. Hydrophobic antibiotics diffuse through the membrane, while hydrophilic